Investigation of the Lactosylation of Whey Proteins by Liquid Chromatography–Mass Spectrometry

CHRISTOPH CZERWENKA,*,† IRENE MAIER,†,§ FRITZ PITTNER,§ AND
WOLFGANG LINDNER†

Institute of Analytical Chemistry and Food Chemistry, University of Vienna, Währinger Strasse 38,
1090 Wien, Austria, and Department of Biochemistry, University of Vienna, 1030 Wien, Austria

Heat treatment of milk induces a reaction between the milk proteins and lactose, resulting in lactosylated protein species. The lactosylation of the two major whey proteins α-lactalbumin and β-lactoglobulin was investigated by reversed phase liquid chromatography−mass spectrometry (LC-MS). Three sample series, consisting of aqueous model solutions of each whey protein separately and in mixture and whole milk, were heated for different time periods, and the progression of the lactosylation reaction was monitored. The observed degrees of lactosylation and the reaction kinetics showed that the lactosylation of β-lactoglobulin was not influenced by the presence of other components, whereas the lactosylation of α-lactalbumin was enhanced in whole milk compared to the aqueous model systems. An in-depth evaluation of the LC-MS data yielded information regarding changes of physicochemical properties of the whey proteins upon lactosylation. Whereas retention time shifts indicated changes in hydrophobicity for both α-lactalbumin and β-lactoglobulin, changes in the charge state distribution denoting conformational alterations were observed only for β-lacto-globulin. The analysis of different liquid and solid milk products showed that the lactosylation patterns of the whey proteins can be used as indicators for the extent of heat treatment.

KEYWORDS: Whey proteins; α-lactalbumin; β-lactoglobulin; lactose; glycation; liquid chromatography−mass spectrometry

INTRODUCTION

Whey proteins are important food proteins not only due to their high nutritional value and their interesting technological properties (*1*) but also from a health perspective, as they constitute potent allergens (*2*). When milk undergoes processing, the technological treatments are likely to result in changes of the whey protein's properties. One of the most common processing steps is heat treatment, which, among other changes, induces the Maillard reaction (*3*). The first step of the Maillard reaction is the formation of a Schiff base between a protein lysine residue and a reducing sugar, which then rearranges to yield an Amadori compound (**Figure 1**). In the case of whey proteins in milk, the reducing sugar is lactose, leading to the formation of lactosylated protein species.

The lactosylation of the two major whey proteins α-lactal-bumin (α-LA) and β-lactoglobulin (β-LG) has been investigated by various techniques, with most studies focusing solely on β-LG. Mass spectrometry, on its own and in combination with liquid chromatography, has been found to be especially useful for monitoring the progression of the lactosylation reaction and

for examining the resulting products in model systems and real-life samples. Several groups have investigated the reaction between β-LG and lactose by electrospray ionization mass spectrometry (ESI-MS) and reversed phase high-performance liquid chromatography−mass spectrometry (RP-HPLC-MS) both in aqueous solution (*4−8*) and in the dry state (*7−9*). Recently, a RP-HPLC-MS study on the lactosylation reaction of α-LA in solution was published as well (*10*). ESI-MS and RP-HPLC-MS were also utilized to investigate the lactosylation of the whey proteins in various consumer and industry milk products (*5, 11−14*). Other techniques that have been employed to examine the lactosylation reaction of the whey proteins include immunochemical methods (*15, 16*) and capillary elec-trophoresis (*6, 17*) addressing β-LG model systems, with the latter technique having also been applied to lactosylated α-LA and β-LG in milk powder samples (*6, 18*). The lysine residues where the reaction with lactose occurs (lactosylation sites) have been determined by analyzing tryptic digests of lactosylated whey protein samples for both α-LA (*12, 14*) and β-LG (*4, 7, 11, 12, 14, 19*). With regard to changes of the functional properties of the whey proteins upon lactosylation, it was shown that the lactosylation of β-LG enhanced its emulsifying and foaming properties (*20−22*).

The studies reported so far on the lactosylation of whey proteins have focused either on more fundamental aspects of

* Corresponding author (telephone +43 1 4277 52322; fax +43 1 4277 9523; e-mail christoph.czerwenka@univie.ac.at).
† Institute of Analytical Chemistry.
§ Department of Biochemistry.

Lactosylation of Whey Proteins

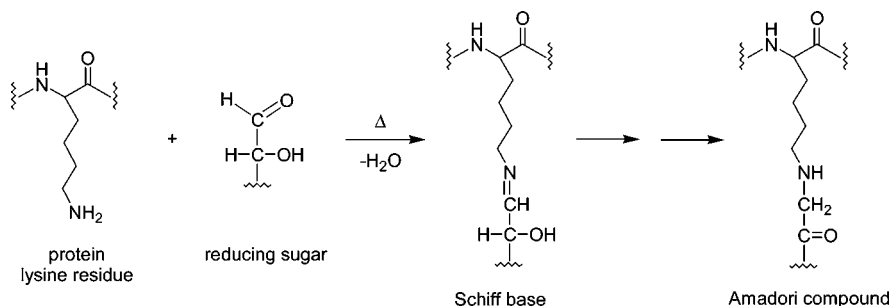*J. Agric. Food Chem.,* Vol. 54, No. 23, 2006   **8875**

Figure 1. Reaction of a protein's lysine residue with a reducing sugar to form an Amadori compound.

the reactions between α-LA or β-LG with lactose (e.g., reaction kinetics, modification sites) using model systems or on the analysis of the lactosylation status in (industrially) processed milk products. However, detailed information on the lactosylation reaction of the whey proteins in their natural environment, that is, in milk, is lacking.

In the present contribution comparative investigations of the lactosylation reaction of the two major whey proteins α-LA and β-LG in different environments (matrices) using liquid chromatography−mass spectrometry (LC-MS) as analysis technique are reported. The formation of Amadori compounds between the whey proteins and lactose upon heat treatment was studied for three sample series, consisting of solution model systems containing one of the whey proteins and lactose (single protein lactosylation), a solution model system in which both whey proteins were incubated together with lactose (protein mixture lactosylation) and a "real world situation" in which whole milk was heated (milk lactosylation). Comparison of the whey protein lactosylation in the different matrices is a novel approach bridging simple model systems with the situation in complex "real world" matrices, which should provide information on whether the lactosylation of a specific whey protein is influenced by the presence of the other whey protein and the other milk components (caseins, etc.), respectively. In studies concerning the thermal denaturation and aggregation of whey proteins the existence of a mutual influence of α-LA and β-LG on each other has been established (*23, 24*). Another focus of the work presented here was an in-depth analysis of the LC-MS data to gain information regarding changes of physicochemical properties of the whey proteins upon lactosylation and thus provide new details of the effects of the lactosylation reaction. Finally, several liquid and solid milk products were analyzed by the LC-MS method to investigate the extent of lactosylation caused by different processing treatments.

MATERIALS AND METHODS

Materials. α-Lactalbumin and lactose monohydrate were purchased from Fluka (Buchs, Switzerland), whereas β-lactoglobulin (mixture of variants A and B) was from Sigma (Steinheim, Germany). Pasteurized whole milk, extended shelf life milk, ultrahigh-temperature-processed (UHT) milk, and sterilized evaporated milk were purchased at a local supermarket, whereas raw milk was acquired from a farmer in Styria (Austria). Whey protein concentrate (WPC) was supplied by the Federal Research Center for Nutrition and Food Kulmbach (Germany). A sachet of coffee whitener was obtained from commercial catering. Gradient grade water (Fluka) was used throughout.

Heat Treatment of Whey Proteins. Aqueous solutions containing α-LA, β-LG, or both proteins and lactose were prepared at concentrations at which these constituents typically are present in milk: 1.5 mg/mL α-LA and/or 3 mg/mL β-LG and 46 mg/mL lactose. These solutions as well as commercial pasteurized whole milk were heated at 60 °C in a water bath. After 0, 2, 4, 8, 16, 24, 48, 72, and 120 h, aliquots were withdrawn and cooled immediately in an ice−water bath. All samples

were stored at −18 °C prior to analysis. For LC-MS analysis the heated whey protein solutions were diluted 1:10. From the heated milk samples whey proteins were isolated using a modified version of the combined lipid extraction and casein precipitation method described by Chen et al. (*25*). To 1 mL of heated milk were added 100 μL of 5% (v/v) acetic acid and 200 μL of dichloromethane, and the mixture was centrifuged for 10 min at 7200 rpm (relative centrifugal force of 3480*g*), yielding three layers with the top one containing the whey proteins. To deplete low molecular weight components an ultrafiltration step was carried out. Three hundred microliters of the top layer was transferred to a centrifugal filter device with a regenerated cellulose membrane with a nominal cutoff mass of 5000 Da (Millipore, Bedford, MA). After centrifugation for 40 min at 8600 rpm (4960*g*), the retentate was further purified by twice adding 100 μL of water and centrifuging for 20 min at 8600 rpm. The final retentate was reconstituted with 1 mL of water to yield the whey protein isolate. For LC-MS analysis these samples were diluted 1:4 with water. Two series of the various samples were heated and processed in parallel.

Isolation of Whey Proteins from Milk Products. Whey proteins were isolated from the liquid milk products and an aqueous solution of the coffee whitener (0.2 g/mL) by lipid removal and casein precipitation. The sample was mixed with the same amount (w/w) of a 0.3 mol/L sodium chloride solution containing 0.2% (w/v) Triton X-100. After extensive homogenization by shaking with glass beads for 1−2 h at room temperature, the solution was centrifuged at 4 °C for 45 min at 16000 rpm (relative centrifugal force of 30360*g*). The aqueous layer was filtered, acidified with 6 mol/L hydrochloric acid to pH 2, and incubated at 37 °C for 20 min. Centrifugation at 4 °C for 20 min at 16000 rpm, followed by filtration, yielded the whey protein isolates. Low molecular weight components were depleted from the whey protein isolates of the liquid milk products by ultrafiltration. Two hundred and fifty microliters of sample was transferred to a centrifugal filter device with a regenerated cellulose membrane with a nominal cutoff mass of 5000 Da (Millipore). After centrifugation for 40 min at 8600 rpm (4960*g*), the retentate was further purified by twice adding 100 μL of water and centrifuging for 20 min at 8600 rpm. The final retentate was reconstituted with 450 μL of water. For LC-MS analysis the whey protein isolates were used directly or diluted 1:2 or 1:5 with water depending on protein concentration. For the WPC sample an aqueous solution of 2 mg/mL was prepared, which was used directly for LC-MS.

LC−MS. The whey protein samples were analyzed by LC-MS using a HP 1100 series HPLC instrument (Agilent Technologies, Waldbronn, Germany) coupled to a PE Sciex API365 triple-quadrupole mass spectrometer (MDS Sciex, Concord, Canada) equipped with an electrospray ion source. The LC separation was carried out on a Supelco Discovery Bio Wide Pore C8 column (150 × 2.1 mm, 3 μm) from Sigma-Aldrich (Vienna, Austria). Elution was performed at a flow rate of 0.25 mL/min with water containing 0.5% (v/v) acetic acid as eluent A and acetonitrile containing 0.5% (v/v) acetic acid as eluent B employing a linear gradient from 35 to 50% B in 16 min. The injection volume was 10 μL, which was increased to 25 μL for samples with very low protein concentrations. The column was thermostated at 40 °C, and the effluent was split approximately 1:100 before entering the mass spectrometer. MS analysis was carried out in the scan mode with positive ionization using an ion spray voltage of 4200 V and scanning from *m/z* 800 to 2200 in 5 s.

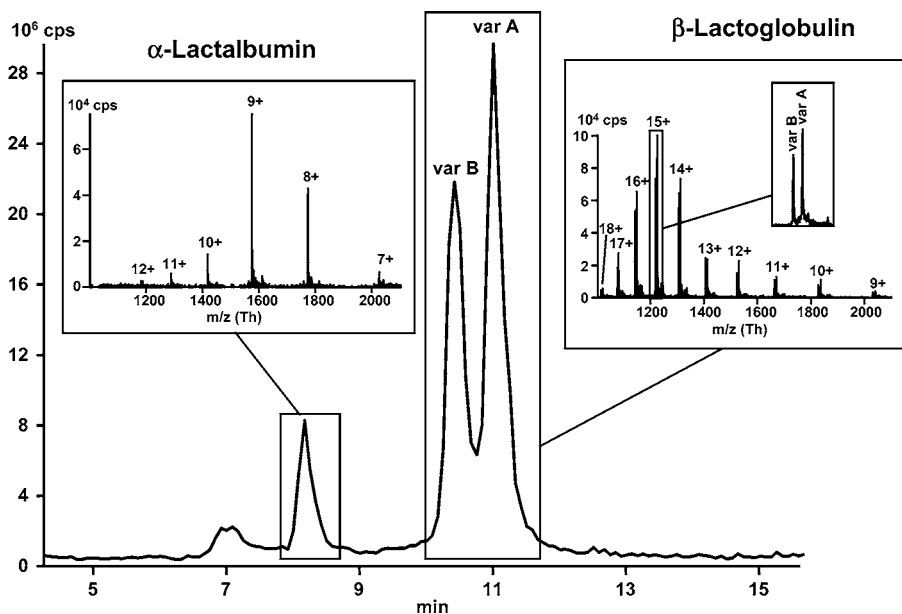**8876** *J. Agric. Food Chem.,* Vol. 54, No. 23, 2006

Czerwenka et al.

Figure 2. Total ion current chromatogram of the separation of the whey proteins α-lactalbumin and β-lactoglobulin. (Insets) Mass spectra of the protein peaks with annotated charge states.

Data Processing. From the total ion chromatograms summed spectra were generated for the protein peaks. As the peaks of the two variants of β-LG showed a partial overlap, a single spectrum containing both variants was generated. The obtained spectra were deconvoluted using MagTran 1.02 software, which is based on a charge state deconvolution algorithm described in ref *26*. For the peak finding, a signal-to-noise threshold of 3 and a mass accuracy of 0.3 Da were set. The deconvolution resulted in the summation of all charge states, avoiding potential problems with changing charge state distributions that would affect an analysis relying on extracted ion chromatograms of selected charge states. The intensities at the respective peak maxima in the deconvoluted spectra were used to determine the degree of lactosylation for each protein as the average number of lactose units bound per protein molecule, which was calculated as follows:

$$\text{degree of lactosylation} = \frac{\sum_{i=0}^{n} i \times \text{intensity}(\text{protein} + i \times \text{lactose})}{\sum_{i=0}^{n} \text{intensity}(\text{protein} + i \times \text{lactose})}$$

RESULTS AND DISCUSSION

Analysis of the Lactosylation of Whey Proteins by LC-MS. The lactosylation of α-LA and β-LG in different solution matrices was studied by heating aqueous solutions containing one or both of these two whey proteins and lactose as well as whole milk at 60 °C for up to 120 h, taking aliquots after nine different incubation periods. The samples will be referred to as "single protein (solution)", "protein mixture (solution)", and "whole milk", respectively, in the following. The concentrations of the proteins and lactose in the single-protein and protein mixture samples were chosen to reflect their respective natural concentrations in whole milk to allow a comparison between the model systems and the "real world" milk sample. The incubation temperature of 60 °C was chosen to provide a sufficient extent of lactosylation while at the same time preventing protein denaturation, which occurs above approximately 65 °C for α-LA and 70 °C for β-LG (*23, 27*).

The heat-treated whey protein solutions and whey protein isolates from the heat-treated milk samples were analyzed by LC-MS combining gradient-elution reversed phase chromatog-

raphy with ESI-MS. A typical chromatogram obtained for the separation of the whey proteins and the mass spectra of the whey protein peaks are shown in **Figure 2**. α-LA and β-LG were cleanly separated from each other within <15 min, and the two variants of β-LG, which differ in two amino acid residues (var A → var B: Asp64 → Gly, Val118 → Ala), were also clearly discernible, although no complete baseline separation was achieved. The mass spectra of α-LA and β-LG (the latter incorporating both variants) exhibited charge state distributions from 7+ to 12+ and from 9+ to 18+, respectively (**Figure 2** insets). Deconvolution of the mass spectra provided the masses of the native (unmodified) proteins and the various lactosylated species.

Exemplary deconvoluted mass spectra are shown in **Figure 3** for samples of β-LG incubated with lactose for 0, 8, 24, and 120 h. In the nonheated sample signals for only native β-LG A and β-LG B were observed at masses that are in excellent agreement with those calculated from the protein sequences (β-LG A, 18363 g/mol; β-LG B, 18277 g/mol). In the sample that had been heated for 8 h, additional signals with a mass increase of 324 Da relative to the respective native β-LG variant were noted. This mass shift corresponds exactly to the addition of one lactose molecule to the protein by formation of a lactulosyllysine Amadori compound (**Figure 1**). In the sample that had been heated for 24 h additionally β-LG species containing two lactosylated lysine residues were detected, whereas the sample that had been incubated with lactose for 120 h showed singly, doubly, and triply lactosylated β-LG species and a shift of the most intensive species from the native form to proteins containing one or two lactose units. Thus, the maximum number of lactose units attached to β-LG and the overall extent of lactosylation increased with the extension of the heat treatment. A similar increase in the extent of lactosylation was observed in the deconvoluted spectra of α-LA, whereby a maximum of two lactose units were attached to the protein under the employed conditions (data not shown).

From the intensities of the various lactosylated and nonlactosylated species in the deconvoluted mass spectra, an overall measure for the extent of the lactosylation reaction was derived by calculating a degree of lactosylation as the average number
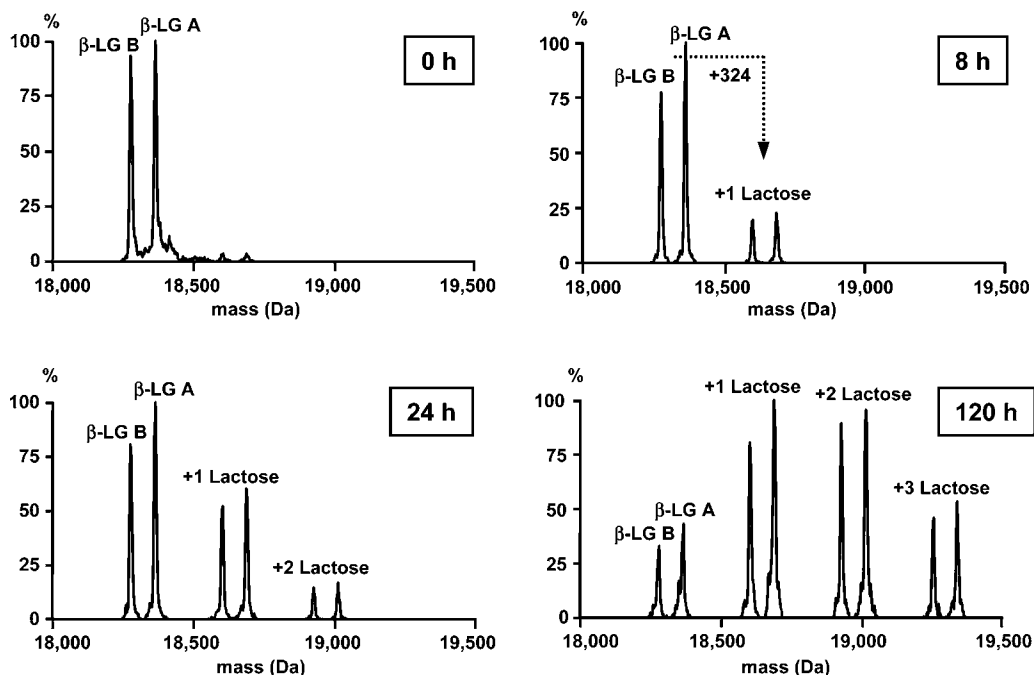
Lactosylation of Whey Proteins

J. Agric. Food Chem., Vol. 54, No. 23, 2006 **8877**

Figure 3. Deconvoluted mass spectra of β-lactoglobulin heated with lactose at 60 °C for 0, 8, 24, and 120 h.

Table 1. Degrees of Lactosylation for Samples of Whey Proteins Heated at 60 °C for Different Time Spans (Mean Values of Two Sample Series Each)

	degree of lactosylation								
	α-lactalbumin			β-lactoglobulin A			β-lactoglobulin B		
time (h)	single protein	protein mixture	whole milk	single protein	protein mixture	whole milk	single protein	protein mixture	whole milk
0	0.000	0.000	0.000	0.041	0.019	0.028	0.037	0.045	0.026
2	0.051	0.000	0.000	0.087	0.083	0.083	0.091	0.089	0.090
4	0.000	0.025	0.000	0.126	0.127	0.125	0.126	0.147	0.148
8	0.098	0.084	0.068	0.216	0.218	0.202	0.207	0.250	0.246
16	0.137	0.149	0.186	0.395	0.395	0.354	0.404	0.421	0.451
24	0.245	0.217	0.218	0.524	0.521	0.587	0.557	0.575	0.695
48	0.403	0.346	0.445[a]	0.949	0.970	1.075[a]	1.012	1.063	0.965[a]
72	0.457	0.557[a]	0.731[a]	1.248	1.115	1.242	1.264	1.191	−[b]
120	0.747	−	1.174[a]	1.544	1.632	−	1.590	1.665	−

[a] Twenty-five microliter injection volume. [b] Spectrum quality not sufficient for deconvolution.

of lactose molecules bound to one protein molecule. This approach assumes identical ionization efficiencies (i.e., response factors) for the unmodified and the variously lactosylated protein species. This assumption is deemed to be valid as the basic protonation site at the lysine's ϵ-amino group remains intact after lactosylation (**Figure 1**). Previous literature reports in which no change in ESI-MS response was found upon glycation of proteins support the validity of this approach (*28*). Moreover, as numerous potential lactosylation sites exist in α-LA and β-LG (*4, 12, 14, 19*), a protein species with a certain number of lactose units attached to it actually corresponds to a mixture of several distinct molecules, in which the lactose is bound to different lysines. Strictly speaking, these individual varieties could also differ in their ionization efficiencies, making any attempt to correctly determine the response factors of all protein species present highly unfeasible.

The degrees of lactosylation obtained for the various samples heated for different time spans are presented in **Table 1**. Whereas both variants of β-LG exhibited a small extent of lactosylation even in the nonheated samples for both the protein solutions prepared from a commercial standard as well as the

whole milk isolate, no traces of lactosylation were observed for α-LA prior to the onset of thermal treatment. With prolonged heating the degree of lactosylation rose from below 0.05 to around 1.6 within 120 h for both variants of β-LG, whereas α-LA consistently showed a much lower degree of lactosylation, reaching the region of an average of one lactose unit per protein molecule after 120 h. The lower extent of lactosylation for α-LA compared to β-LG can partly be explained by the different numbers of lysine residues, which amount to 15 for β-LG (both variants) and to 12 for α-LA. However, even if one takes this difference in the number of potential lactosylation sites into account and compares the average number of lactose units bound per lysine residue, α-LA still shows a consistently lower degree of reaction with lactose than β-LG (e.g., lactose units per lysine residue after 48 h of heat treatment: α-LA $\approx$ 0.033, β-LG $\approx$ 0.067). This finding suggests that—on average—the lysine residues in α-LA are less accessible for the formation of a Schiff base with lactose than those in β-LG. In contrast, the degrees of lactosylation between variants A and B of β-LG are generally comparable, although it was noted that β-LG B exhibited slightly higher values in the majority of data points. This observation
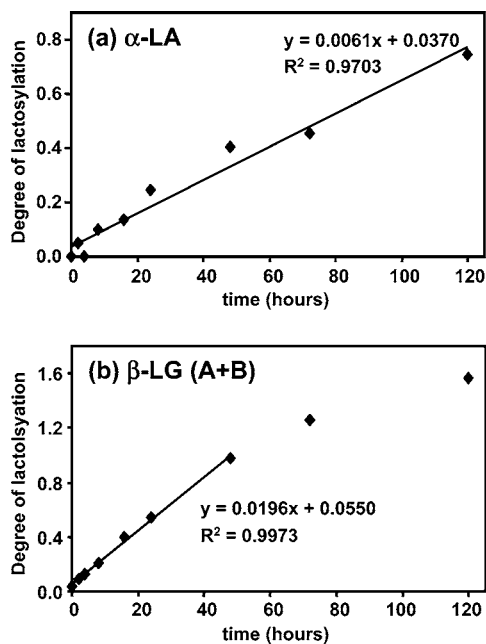
Figure 4. Kinetics of lactosylation of (**a**) α-lactalbumin and (**b**) β-lactoglobulin (sum of variants A and B). Data are from single-protein solution experiments.

is in good agreement with a study on dry-state glycation of β-LG A and B (*9*). Therefore, the lactosylation of β-LG may be characterized by the results obtained for the sums of the variants A and B.

The progression of the lactosylation of the whey proteins with prolonged heat treatment can be succinctly summarized in the form of kinetic plots, which are depicted in **Figure 4**. For α-LA the degree of lactosylation showed a linear increase over the entire 120 h of heat treatment. In contrast, the lactosylation of β-LG proceeded linearly only for 48 h. Although longer incubation times further increased the average number of lactose units bound to β-LG, the reaction occurred at a decelerated speed. Because lactose is present in huge excess compared to β-LG (molar ratio of approximately 800:1) this kinetics suggests that the lactosylation reactions of β-LG species already carrying one or more lactose units occur at slower rates than that of native β-LG.

Comparison of the Lactosylation of Whey Proteins in Different Matrices. A comparison of the reaction of α-LA and β-LG with lactose in different matrices (aqueous single-protein solution, aqueous protein mixture solution, and whole milk) was carried out to assess whether the lactosylations of these whey proteins are influenced by the presence of other components, that is, the other whey protein and other milk constituents (caseins, lipids, etc.), respectively. Although the samples consisting of aqueous solutions could be analyzed by LC-MS directly after dilution, an isolation procedure was necessary for the milk samples to remove lipids and caseins. This workup may lead to losses of the whey proteins, but it can be expected that the recovery rates will be identical for native and lactosylated whey proteins. In this context it was noted that the protein concentrations in the whey isolates from the milk samples that had been incubated for longer periods ($\geq$48 h) were low compared to the other samples (aqueous whey protein solutions and milk heated for shorter periods), resulting in the need for larger injection volumes and sometimes poor data quality prohibiting successful deconvolution of the mass spectra. The reason for this observation may be attributed to reactions of
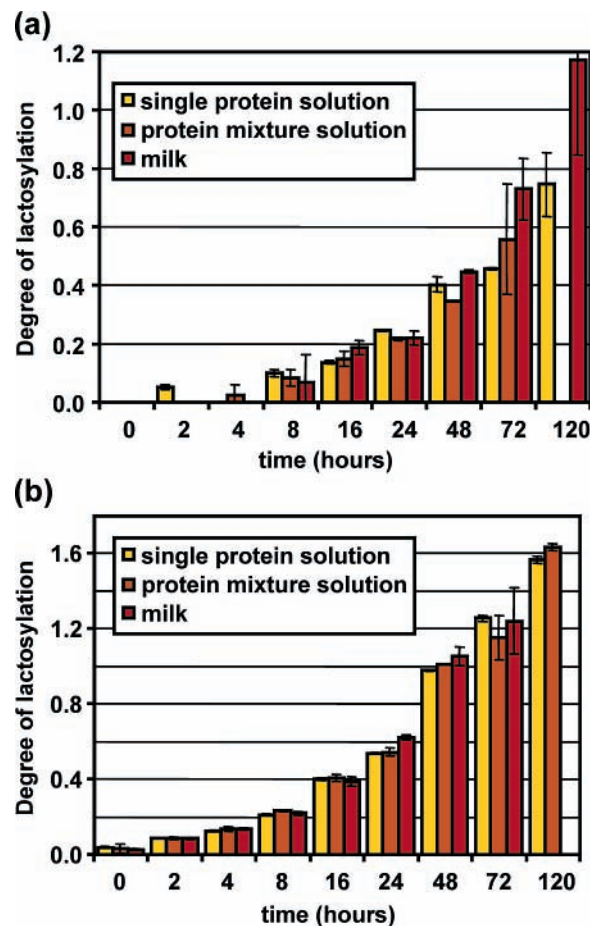

Figure 5. Comparison of degrees of lactosylation for (**a**) α-lactalbumin and (**b**) β-lactoglobulin (sum of variants A and B) from single-protein solutions, protein mixture solution, and whole milk. Error bars indicate standard deviations.

the whey proteins with caseins or other major milk constituents, resulting in the formation of high molecular weight aggregates.

The degrees of lactosylation for the three sample series obtained after various periods of heat treatment are juxtaposed in **Figure 5**. Comparison of the single-protein samples with the protein mixture samples revealed no significant differences between these two series for both α-LA and β-LG. Whereas the results for β-LG were very similar for all incubation periods, the degrees of lactosylation varied somewhat for α-LA, especially for the samples that had undergone longer heat treatments; however, these variations were scattered and showed no distinct trend. Therefore, neither of the two whey proteins α-LA and β-LG influences the other's lactosylation in an aqueous solution. With regard to the whey proteins isolated from heated milk, it was found that the degrees of lactosylation of β-LG corresponded well with those from the aqueous protein solutions. Thus, none of the milk constituents seems to significantly influence the reaction of β-LG with lactose. In contrast to this, α-LA showed considerably higher degrees of lactosylation in the milk samples than in the protein solution samples upon prolonged heat treatment. It seems that the lactosylation of α-LA is promoted by the presence of at least one of the other milk constituents. One may speculate that α-LA undergoes interactions with one of these compounds (e.g., the caseins), which enhance its susceptibility toward a reaction with lactose. The results for β-LG would then indicate that this whey protein either does not engage in such interactions or that—in

Lactosylation of Whey Proteins

J. Agric. Food Chem., Vol. 54, No. 23, 2006 **8879**

Table 2. Parameters of the Linear Regressions $y = a \cdot x + b$ of the Degree of Lactosylation versus Heating Time Obtained for α-Lactalbumin and β-Lactoglobulin (Sum of Variants A and B) from Different Samples

	whey protein source	a	b	r^2
α-lactalbumin[a]	single-protein solution	0.0067 ± 0.0007	0.0281 ± 0.0222	0.9411
	protein mixture solution	0.0076 ± 0.0003	0.0068 ± 0.0103	0.9897
	whole milk	0.0102 ± 0.0004	-0.0147 ± 0.0115	0.9927
β-lactoglobulin[b]	single-protein solution	0.0196 ± 0.0005	0.0550 ± 0.0098	0.9973
	protein mixture solution	0.0202 ± 0.0005	0.0551 ± 0.0101	0.9973
	whole milk	0.0215 ± 0.0007	0.0485 ± 0.0159	0.9941

[a] Data from samples heated for 0–72 h ($n = 8$). [b] Data from samples heated for 0–48 h ($n = 7$).

the more likely case that they do exist—these interactions do not affect its lactosylation.

These strikingly different lactosylation behaviors of α-LA and β-LG in heated milk samples is also reflected by the slopes of the regression curves that were calculated for the linear ranges of the kinetic plots (degree of lactosylation versus heating time) for the various sample series and which are presented in **Table 2**. Whereas the parameters of the linear regressions for β-LG show practically no significant differences between the sample series (the slope of the regression curve of the milk samples being just slightly higher than those of the protein solution samples), pronounced differences exist for α-LA. The slopes of the linear regressions are comparable for the single-protein and the protein mixture sample series, whereas the slope of the regression for the milk samples is significantly higher than those of the former two.

This comparison of the extents of lactosylation of whey proteins induced upon heat treatment in different matrices showed that model systems consisting of aqueous solutions do not necessarily correctly reflect the situation and modification processes in "real world" environments (matrices). Moreover, it was found that the deviations between model systems and real-life samples can vary considerably for different proteins. Although highly simplified model systems are certainly useful for assessing fundamental questions without the influence of other, potentially interacting, components, comparable systematic investigations of "real world" samples, which still have a rather low level of complexity (as represented by whole milk here), may contribute to the understanding of modification processes of proteins in complex food samples.

Changes of Physicochemical Properties of Whey Proteins upon Lactosylation. A substantial variety of information can be obtained from an in-depth evaluation of the data provided by LC-MS analysis (chromatographic parameters and time-resolved mass spectra). A detailed investigation of the LC-MS data acquired for the various lactosylated whey protein samples was carried out to gain information regarding changes of the physicochemical properties of α-LA and β-LG upon lactosylation.

First, the retention times of the various protein species having no, one, two, or three lactose units attached to them were investigated, whereby the discrimination between the different protein species was made entirely upon the number of bound lactose units. Thus, variants resulting from different lysine binding sites were treated together (see also above). The chromatographic peaks of the protein species with different numbers of lactose units were obtained by generating extracted ion chromatograms for the charge state with the highest intensity (9+ for α-LA and 15+ for β-LG). The overlaid chromatographic traces of the various protein species are shown in **Figure 6** for α-LA and β-LG A (β-LG B gave equivalent results; data not shown). The same trend in retention time was observed for
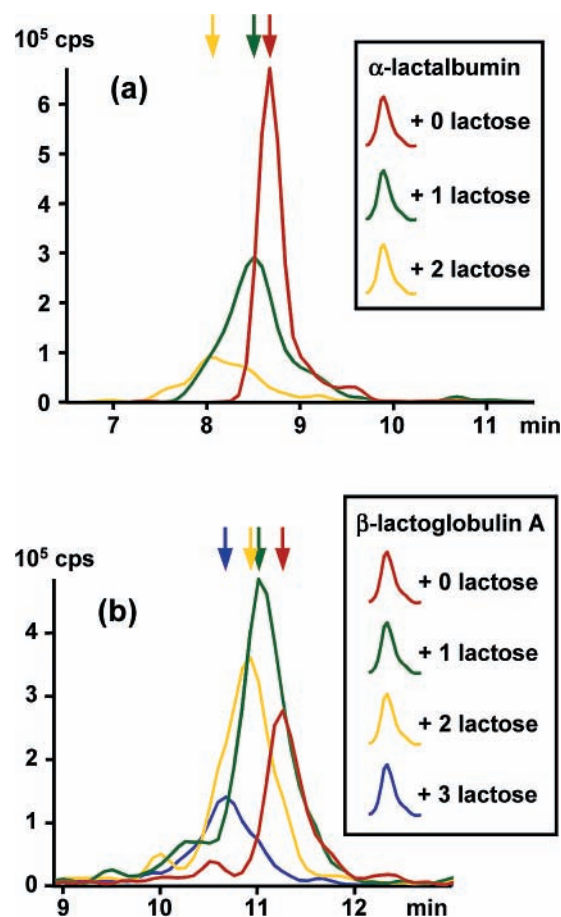

Figure 6. Liquid chromatographic retentions of lactosylated whey proteins: extracted ion chromatograms of (**a**) α-lactalbumin species (charge state 9+) and (**b**) β-lactoglobulin A species (charge state 15+). Arrows indicate respective chromatographic peak maxima.

both whey proteins. With an increasing number of lactose units attached to the protein, its retention decreased significantly. The shift in retention time amounted to approximately 0.5 min between the native protein and the species carrying the most lactose units for both α-LA and β-LG. As a reduction in retention time in reversed phase chromatography corresponds to a decrease in overall hydrophobicity of the interacting analyte surface, the extracted ion chromatograms indicate that the addition of highly hydrophilic lactose moieties, which occurs at the lysine residues exposed at the protein's surface, led to a reduction of the protein's overall hydrophobicity. The higher the number of attached lactose units was, the less hydrophobic the protein became. The broad and in some cases asymmetric shape of the chromatographic peaks of the highly lactosylated protein species (e.g., α-LA + 2 lactose; see **Figure 6**) possibly results from the variety of individual protein species with
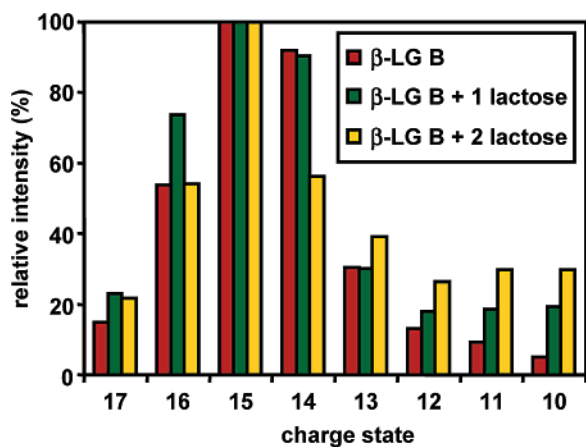
Figure 7. Mass spectrometric charge state distributions of different lactosylated β-lactoglobulin B species from a sample of β-lactoglobulin heated with lactose for 16 h.

different lactose binding sites that are summarized in these peaks and which may differ subtly in their retention characteristics.

The second characteristic of the variously lactosylated whey proteins that was evaluated was their charge state distribution (CSD). The CSD of a protein depends on the number of accessible ionization sites, which in turn is influenced by the protein's conformation. Therefore, changes in the CSD can provide information regarding conformational changes induced by structural modifications (*29*).

Thus, the relative intensities of each single charge state were determined for the protein species having different numbers of attached lactose units (representing again the sum of species with different lysine binding sites). As an example, the plot of the relative intensities of the different charge states for β-LG B is shown in **Figure 7**. Whereas the maximum of the CSD was the same for the native β-LG as well as for the β-LG species carrying one and two lactose units (namely, the 15+ charge state), the shape of the CSDs varied. The CSD of the native β-LG exhibited a bell-shaped curve, whereas both lactosylated β-LG species showed a "tail" of constant relative intensities from the 10+ to the 12+ charge state. The differences between the CSDs come to light most cleary if one examines the deviations between the relative intensities of the differently lactosylated β-LG species and expresses them as relative differences. For example, the relative intensities of the 11+ and 10+ charge states increase by factors of 3 and 6, respectively, between the native and the doubly lactosylated β-LG species, whereas the relative differences are below 50% for the higher charge states (14+ to 17+). Similar observations were made for β-LG A, whereas, in contrast, no distinct changes upon lactosylation were noted for the CSD of α-LA (data not shown).

An alternative way of characterizing a CSD is the calculation of an average charge state (intensity-weighted mean of all observed charge states). The average charge states obtained for the variously lactosylated species of α-LA and β-LG are given in **Table 3**. Although the average charge state remained unaffected by lactosylation for α-LA, significant decreases occurred with increasing lactosylation for both variants of β-LG, with the effect being more pronounced for variant B.

It is important to note again that lactosylation of a protein by the formation of an Amadori compound does not change the number of available ionization sites—the primary ϵ-amino group of lysine is converted into a secondary amine (**Figure 1**), which still may be protonated. However, the lactose moiety may hamper the access of the proton to the amine, reducing

Table 3. Average Mass Spectrometric Charge States of Different Lactosylated Whey Protein Species

lactose units attached	average charge state[a]		
	α-lactalbumin	β-lactoglobulin A	β-lactoglobulin B
0	8.59 ± 0.05	14.48 ± 0.05	14.45 ± 0.07
1	8.62 ± 0.07	14.32 ± 0.08	14.26 ± 0.07
2		14.17 ± 0.15	13.91 ± 0.16

[a] Charge states are given as mean value ± standard deviation ($n = 6$, two protein samples each heated with lactose at 60 °C for 16, 24, and 48 h).

Table 4. Degrees of Lactosylation for Different Liquid and Solid Milk Products

milk product	degree of lactosylation[a]		
	α-lactalbumin	β-lactoglobulin A	β-lactoglobulin B
raw milk	0.000 ± 0.000	0.116 ± 0.014	0.118 ± 0.015
pasteurized milk	0.000 ± 0.000	0.026 ± 0.044	0.055 ± 0.048
extended shelf life milk	0.000 ± 0.000	0.014 ± 0.024	0.018 ± 0.032
ultrahigh-temperature milk	0.329 ± 0.289	0.651 ± 0.180	0.698 ± 0.055
evaporated milk	_[b]	_[b]	_[b]
whey protein concentrate	0.394 ± 0.073	0.564 ± 0.023	0.606 ± 0.024
coffee whitener	_[c]	_[c]	_[c]

[a] Degrees of lactosylation are given as mean value ± standard deviation ($n = 3$). [b] No whey proteins detected. [c] Levels of whey proteins too low to allow deconvolution.

the probability of it becoming charged. The results presented above show that lactosylation has a significant influence on the CSD of β-LG, shifting it toward lower charge states, whereas the CSD of α-LA remained unaffected. The obtained CSDs indicate that lactosylation induced a slight change in the conformation of β-LG toward a more compact (folded) structure (*29*), but it had no influence on the conformation of α-LA. The different influences of lactosylation on the CSDs of α-LA and β-LG may be explained by different structural changes of the proteins upon lactosylation. The effects of the addition of a lactose moiety on the complex network of interactions that is responsible for a protein's three-dimensional structure will depend on the initial structure and on the environment of the lactosylated lysine residue. The multitude of potential lactosylation sites results in the observed CSD changes representing an average for several variants, which prohibits a clear assignment of these observations to certain structural features. The finding concerning β-LG is consistent with a previous literature report (*9*) and agrees well with the observation noted above that the addition of further lactose units to already lactosylated β-LG species proceeds at a slower rate than the lactosylation reaction of native β-LG. It would be expected that a more compact structure of an already lactosylated β-LG species may impede the addition of a further lactose unit.

Lactosylation of Whey Proteins in Various Milk Products. The developed LC-MS method was also applied to the analysis of whey protein isolates of several liquid and solid milk products to investigate the lactosylation caused by different processing treatments. Milks that had undergone an increasing extent of heat treatment (from raw to evaporated milk) as well as a whey protein concentrate (WPC) and a coffee whitener were analyzed. The degrees of lactosylation obtained for these samples are given in **Table 4**, and examples of the deconvoluted spectra acquired are shown in **Figure 8**.

In the milk samples that had undergone no or mild heat treatment (raw, pasteurized, and extended shelf life milk) α-LA showed no lactosylation, whereas the degrees of lactosylation

Lactosylation of Whey Proteins

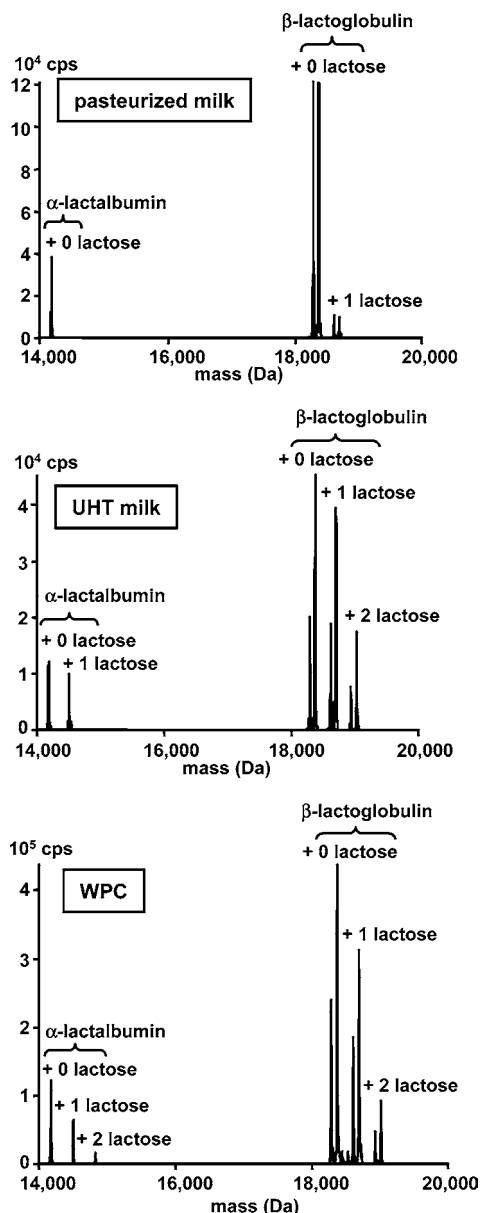*J. Agric. Food Chem.,* Vol. 54, No. 23, 2006   **8881**

Figure 8. Deconvoluted mass spectra of whey proteins isolated from different milk products (each spectrum is a combination of the spectra obtained for the chromatographic peaks of α-lactalbumin and β-lactoglobulin).

of β-LG were rather low and of similar magnitude. In contrast, UHT milk showed extensive lactosylation for both α-LA and β-LG, with a maximum of one and two lactose units attached, respectively. The intense heat treatment used in the production of evaporated milk obviously effected pronounced changes of the whey proteins, as they could not be detected. In the group of solid milk products the WPC exhibited substantial lactosylation. The degrees of lactosylation of both α-LA and β-LG were comparable to those observed for the UHT milk; however, it was noted that the WPC exhibited a doubly lactosylated α-LA species, which was not observed for the UHT milk (**Figure 8**). Although whey proteins were detected in the isolate from the coffee whitener, their levels were too low to enable deconvolution of the mass spectra.

The results obtained for the various milk products show that the degrees of lactosylation of the two major whey proteins α-LA and β-LG can serve as indicators for the extent of heat

treatment the milk products have experienced. Whereas the extent of lactosylation of one of these proteins is sufficient to determine the intensity of the applied heat treatment, the investigation of the lactosylation of both proteins can provide additional information for characterizing heat-treated milk products (different patterns of lactosylation of α-LA for the UHT milk and the WPC, which had both experienced extensive heat treatment). However, further investigations on this topic are necessary.

In conclusion, the LC-MS analysis of the lactosylation of the whey proteins α-LA and β-LG, resulting from different heat treatments, in various matrices has shown the usefulness of this methodology both for obtaining information on fundamental issues such as reaction kinetics and physicochemical changes and for characterizing the processing of commercial milk products. Although other constituents present in whole milk were found to influence the lactosylation reaction of α-LA compared to aqueous model systems, no difference was observed for β-LG. An in-depth evaluation of the multifaceted LC-MS data showed a reduction of the proteins' hydrophobicities upon lactosylation, resulting in retention time shifts and changes in the charge state distributions of both β-LG variants, pointing toward conformational changes. Characteristic differences between the lactosylation of α-LA and β-LG in various liquid and solid milk products showed the applicability of the method for monitoring industrial heat treatments.

ABBREVIATIONS USED

α-LA, α-lactalbumin; β-LG, β-lactoglobulin; CSD, charge state distribution; ESI-MS, electrospray ionization mass spectrometry; LC-MS, liquid chromatography–mass spectrometry; RP-HPLC-MS, reversed phase high-performance liquid chromatography–mass spectrometry; UHT, ultrahigh-temperature-processed; WPC, whey protein concentrate.

ACKNOWLEDGMENT

LITERATURE CITED

(1) de Wit, J. N. Nutritional and functional characteristics of whey proteins in food products. *J. Dairy Sci.* **1998**, *81*, 597−608.

(2) Wal, J. M. Allergy review series II: an update on allergens: cow's milk allergens. *Allergy* **1998**, *53*, 1013−1022.

(3) van Boekel, M. A. J. S. Effect of heating on Maillard reactions in milk. *Food Chem.* **1998**, *62*, 403−414.

(4) Morgan, F.; Léonil, J.; Mollé, D.; Bouhallab, S. Nonenzymic lactosylation of bovine β-lactoglobulin under mild heat treatment leads to structural heterogeneity of the glycoforms. *Biochem. Biophys. Res. Commun.* **1997**, *236*, 413−417.

(5) Leonil, J.; Molle, D.; Fauquant, J.; Maubois, J. L.; Pearce, R. J.; Bouhallab, S. Characterization by ionization mass spectrometry of lactosyl β-lactoglobulin conjugates formed during heat treatment of milk and whey and identification of one lactose-binding site. *J. Dairy Sci.* **1997**, *80*, 2270−2281.

(6) Jones, A. D.; Tier, C. M.; Wilkins, J. P. G. Analysis of the Maillard reaction products of β-lactoglobulin and lactose in skimmed milk powder by capillary electrophoresis and electrospray mass spectrometry. *J. Chromatogr. A* **1998**, *822*, 147−154.

(7) Morgan, F.; Bouhallab, S.; Mollé, D.; Henry, G.; Maubois, J. L.; Leónil, J. Lactolation of β-lactoglobulin monitored by electrospray ionisation mass spectrometry. *Int. Dairy J.* **1998**, *8*, 95−98.

8882 *J. Agric. Food Chem.,* Vol. 54, No. 23, 2006

Czerwenka et al.

(8) French, S. J.; Harper, W. J.; Kleinholz, N. M.; Jones, R. B.; Green-Church, K. B. Maillard reaction induced lactose attachment to bovine β-lactoglobulin: electrospray ionization and matrix-assisted laser desorption/ionization examination. *J. Agric. Food Chem.* **2002**, *50*, 820−823.

(9) Fenaille, F.; Morgan, F.; Parisod, V.; Tabet, J. C.; Guy, P. A. Solid-state glycation of β-lactoglobulin monitored by electrospray ionisation mass spectrometry and gel electrophoresis techniques. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 1483−1492.

(10) Lund, M. N.; Olsen, K.; Sørensen, J.; Skibsted, L. H. Kinetics and mechanism of lactosylation of α-lactalbumin. *J. Agric. Food Chem.* **2005**, *53*, 2095−2102.

(11) Fogliano, V.; Monti, S. M.; Visconti, A.; Randazzo, G.; Facchiano, A. M.; Colonna, G.; Ritieni, A. Identification of a β-lactoglobulin lactosylation site. *Biochim. Biophys. Acta* **1998**, *1388*, 295−304.

(12) Siciliano, R.; Rega, B.; Amoresano, A.; Pucci, P. Modern mass spectrometric methodologies in monitoring milk quality. *Anal. Chem.* **2000**, *72*, 408−415.

(13) Hau, J.; Bovetto, L. Characterisation of modified whey protein in milk ingredients by liquid chromatography coupled to electrospray ionisation mass spectrometry. *J. Chromatogr. A* **2001**, *926*, 105−112.

(14) Marvin, L. F.; Parisod, V.; Fay, L. B.; Guy, P. A. Characterization of lactosylated proteins of infant formula powders using two-dimensional gel electrophoresis and nanoelectrospray mass spectrometry. *Electrophoresis* **2002**, *23*, 2505−2512.

(15) Matsuda, T.; Kato, Y.; Watanabe, K.; Nakamura, R. Direct evaluation of β-lactoglobulin lactosylation in early Maillard reaction using an antibody specific to protein-bound lactose. *J. Agric. Food Chem.* **1985**, *33*, 1193−1196.

(16) Morgan, F.; Vénien, A.; Bouhallab, S.; Mollé, D.; Léonil, J.; Peltre, G.; Levieux, D. Modification of bovine β-lactoglobulin by glycation in a powdered state or in an aqueous solution: immunochemical characterization. *J. Agric. Food Chem.* **1999**, *47*, 4543−4548.

(17) Otte, J.; Larsen, K. S.; Bouhallab, S. Analysis of lactosylated β-lactoglobulin by capillary electrophoresis. *Int. Dairy J.* **1998**, *8*, 857−862.

(18) Guyomarc'h, F.; Warin, F.; Muir, D. D.; Leaver, J. Lactosylation of milk proteins during the manufacture and storage of skim milk powders. *Int. Dairy J.* **2000**, *10*, 863−872.

(19) Fenaille, F.; Morgan, F.; Parisod, V.; Tabet, J. C.; Guy, P. A. Solid-state glycation of β-lactoglobulin by lactose and galactose: localization of the modified amino acids using mass spectrometric techniques. *J. Mass Spectrom.* **2004**, *39*, 16−28.

(20) Nacka, F.; Chobert, J. M.; Burova, T.; Leónil, J.; Haertlé, T. Induction of new physicochemical and functional properties by the glycosylation of whey proteins. *J. Protein Chem.* **1998**, *17*, 495−503.

(21) Groubet, R.; Chobert, J. M.; Haertlè, T.; Nicolas, M. G. Functional properties of milk proteins glycated in mild conditions. *Sci. Aliments* **1999**, *19*, 423−438.

(22) Chevalier, F.; Chobert, J. M.; Popineau, Y.; Nicolas, M. G.; Haertlé, T. Improvement of functional properties of β-lactoglobulin glycated through the Maillard reaction is related to the nature of the sugar. *Int. Dairy J.* **2001**, *11*, 145−152.

(23) Boye, J. I.; Alli, I. Thermal denaturation of mixtures of α-lactalbumin and β-lactoglobulin: a differential scanning calorimetric study. *Food Res. Int.* **2000**, *33*, 673−682.

(24) Schokker, E. P.; Singh, H.; Creamer, L. K. Heat-induced aggregation of β-lactoglobulin A and B with α-lactalbumin. *Int. Dairy J.* **2000**, *10*, 843−853.

(25) Chen, R. K.; Chang, L. W.; Chung, Y. Y.; Lee, M. H.; Ling, Y. C. Quantification of cow milk adulteration in goat milk using high-performance liquid chromatography with electrospray ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 1167−1171.

(26) Zhang, Z.; Marshall, A. G. A Universal algorithm for fast and automated charge state deconvolution of electrospray mass-to-charge ratio spectra. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 225−233.

(27) Iametti, S.; De Gregori, B.; Vecchio, G.; Bonomi, F. Modifications occur at different structural levels during the heat denaturation of β-lactoglobulin. *Eur. J. Biochem.* **1996**, *237*, 106−112.

(28) Yeboah, F. K.; Yaylayan, V. A. Analysis of glycated proteins by mass spectrometric techniques: qualitative and quantitative aspects. *Nahrung/Food* **2001**, *45*, 164−171.

(29) Grandori, R. Electrospray-ionization mass spectrometry for protein conformational studies. *Curr. Org. Chem.* **2003**, *7*, 1589−1603.

JF061646Z